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FEMS Microbiology Letters 196 (2001) 165–170

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Taxon-specific oligonucleotide primers for detection of two ancient endomycorrhizal fungi, *Glomus occultum* and *Glomus brasilianum*

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Received 22 January 2001; accepted 31 January 2001

Abstract

A unique oligonucleotide pair, GOCC56:GOCC427, was designed that correctly primed specific amplification of a ~370-bp sequence spanning the ITS and 5.8S rDNA regions of *Glomus occultum* and *Glomus brasilianum*. In addition, this primer pair successfully detected *G. occultum* and *G. brasilianum* DNA in nested PCR using a primary PCR product amplified from highly diluted extracts of colonized corn (*Zea mays*) roots using modified ITS1:ITS4 primers. A second primer pair, GBRAS86:GBRAS388, primed specific amplification of a ~200-bp sequence spanning the ITS and 5.8S rDNA regions present only in *G. brasilianum* and *Glomus* strain GR582. Combined use of both primer pairs provides the means to detect and differentiate two ancient endomycorrhizal species, *G. occultum* and *G. brasilianum*, undetectable by standard root staining procedures. Sequence analysis showed that the purported *G. occultum* strain GR582 is likely a strain of *G. brasilianum*.

Keywords: Arbuscular mycorrhizal fungus; Internal transcribed spacer; ITS; Ribosomal DNA; rDNA; PCR primer

1. Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with roots of more than 90% of all taxonomic groups of terrestrial plants worldwide. Recently, molecular approaches, involving polymerase chain reaction (PCR), nucleotide sequencing, single-stranded conformational analysis, and sequence-based phylogenetic analysis, have been used to improve identification and taxonomy and to assess diversity within this group of fungi, i.e., Glomales [1–7]. As a result, subgroup-specific primers and taxon-specific probes have been developed for a few of the 150 recognized species of these fungi [8–12].

The purpose of this study was to develop PCR primers for use in detecting *Glomus occultum*, one of the most widespread but difficult to detect AMF (in roots with standard staining protocols). A means to clearly distinguish *G. occultum* from closely related taxa, such as *Glomus brasilianum*, or morphologically similar taxa, e.g., *Glomus diaphanum*, are specifically needed for diversity studies. Based on previous success with development of taxon-specific primers for *Glomus mosseae* and *Glomus etunicatum*, we focused on the ITS regions of rDNA.

2. Materials and methods

2.1. Fungal isolates and spore collection

The 45 isolates comprising 27 different AMF taxa used in this study were as follows with INVAM designations (GenBank accession number/number of spores extracted): *G. brasilianum* WV 224 (AF165920 to AF165922/500); *G. brasilianum* WV212 (400); *G. brasilianum* WV215A (250); *G. brasilianum* ITH43-MD (AF165918, AF165919/300); *Glomus caledonium* UK301(1); *Glomus claroideum* SC186 (AF004687, AF004688/250); *G. claroideum* MD125 (U94715, U94716/70); *Glomus clarum* WV751(235); *Glomus constrictum* MDFSP-11 (30); *Glomus deserticola* CA113 (250); *G. diaphanum* WV579B (350); *G. diaphanum* DW47 (200); *G. etunicatum* NE108A (150); *Glomus fasciculatum* MD212 (100); *Glomus fistulosum* DN987 (190); *Glomus geosporum* MD124 (AF197918/100); *Glomus ger-*

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0378-1097/01/$20.00
PII: S0378-1097(01)00063-5

demannii AU215A (20); *Glomus intraradices* FL208 (AF004681/100); *Glomus microaggregatum* MDFSP-7 (100); *G. mosseae* FL156 (U49265/50); *G. occultum* CL383 (AF005064, AF005065, AF005481/1000); *G. occultum* CL700C (AF004677 to AF004679/1000); *G. occultum* FL703 (AF005062/600); *G. occultum* IA702 (AF004675, AF004676/1000); *G. occultum* DW46-20 (400); *G. occultum* VZ103C (250); *G. occultum* SF123 (150); *G. occultum* GR582 (U81987/500); *Glomus sinuosum* MD126 (36); *Glomus spercum* NB106A (650); *G. spercum* SC151 (200); *G. spercum* AZ420B (450); *G. spercum* TX144 (350); *Glomus viscosum* MD213; *Gigaspora gigantea* PA149 (AF004685, AF004684/8); *Glomus rosea* FL105 (AF004696 to AF004699/1); *G. rosea* UT102 (20); *Acaulospora gerdmannii* FL130A (20); *Acaulospora lacunosa* NH102 (AF006512/60); *Acaulospora scrobiculata* MDSBT-10 (200); *Entrophospora columbiana* GA 101(250); *Entrophospora contigua* WV201 (AF005060, AF005061/20); *Entrophospora infrequens* NY349 (U94713, U94714/20); *Scutellospora coralloidea* CA 260 (AF004686/1). *Pythium ultimum* PuZ1 and *Endogone pisiformis* EP1 were non-mycorrhizal fungus controls and *Zea mays* (corn), was the plant DNA control. Individual INVAM (International Culture Collection of Vesicular Arbuscular and AMF, Morgantown, WV, USA) isolates of AMF were grown with *Z. mays* in pot cultures containing soil:sand (1:1, v:v); spores were harvested, and cleaned as previously described [10,11], or they were obtained directly from INVAM.

2.2. DNA extraction

Spores were crushed in Eppendorf tubes using disposable (single use) autoclaved, polycarbonate micropestles. Spore crushes were resuspended in 10 volumes of PCR grade water and a one-third volume of Chelex 100 resin solution (20% w/v) before 15 s sonication in a jewelry cleaning unit (Model 77, Electromation Components Corp., NY, USA), then freeze-thawed (−20°C, room temperature) three times. Preparations were stored at −20°C; all dilutions were made with PCR grade water. Corn roots (150 mg) were frozen (−20°C), ground to fine powder with liquid nitrogen in a mortar and pestle (−80°C), and extracted using a DNAzol ES DNA Extraction kit (Molecular Research Center, Cincinnati, OH, USA).

2.3. Oligonucleotides, PCR conditions and product analysis

Oligonucleotides were synthesized commercially and used unpurified. Sequences of ITS4*Pst* and ITS1*Kpn* and their location relative to ITS and 5.8S regions were described previously [11]; other oligonucleotides are listed in Table 1.

PCRs were performed with an automated temperature cycling instrument with mixtures containing the following (final concentrations): 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 250 μM each of the four deoxynucleotide triphosphates (dNTPs), 0.25 μM (each) primer, 50 units ml^{-1} of Amplitaq Gold DNA polymerase (PE Applied Biosystems, CA, USA) and template DNA. Reaction mixtures contained a 1:10 volume of diluted template DNA (dilutions ranged from 1:10 to 1:200 for glomalean templates and 1:100 to 1:1000 for non-mycorrhizal control templates). Dilutions of *G. occultum* template DNA that produced adequate product yields with the ITS1*Kpn*: ITS4*Pst* primers also produced good yields when amplified with GOCC56 and GOCC427. Reaction mixtures used for detecting *G. occultum* in root fragment preparations contained dilutions of root extract DNA ranging from 1:100 to 1:500.

PCR components were assembled on ice, then transferred to a thermal cycler block pre-heated to 95°C (to reduce non-specific priming). Reactions were initially heated for 9 min at 95°C to activate the enzyme, and then those with ITS primers were subjected to 40 cycles of 30 s at 95°C, 45 s at 55°C, and 60 s at 72°C; those with primer pairs GOCC56:GOCC427 or GBRAS86: GBRAS388 had 35 cycles of 60 s at 95°C, 60 s at 53°C, and 60 s at 72°C; all reactions concluded with a final extension step of 72°C, 5 min.

2.4. DNA cloning and sequencing

DNA products from 100 μl PCRs using ITS1*Kpn* and ITS4*Pst* primers were diluted and washed twice with 10-fold volumes of water in Centricon-30 units (Amicon, Inc., Boston, MA, USA). After digestion with *Kpn*I and *Pst*I and electrophoresis, products were purified from 0.8% agarose gel using a GeneClean kit (Bio101, La Jolla, CA, USA), and ligated with 0.25 μg of pUC19 DNA that had been digested with *Kpn*I and *Pst*I. Ligated products were used to transform electrocompetent cells of *Escherichia coli* DH5α. Alternatively, some PCR products were purified and cloned directly into pT7Blue3 and introduced into Novablue cells according to the manufacturer's directions (Novagen). An alkaline lysis miniprep procedure [13] was used to identify transformants containing plasmid inserts of the correct size (590 bp). Initial plasmid DNA preparations for sequencing were purified by alkaline lysis with at least one PEG precipitation step to remove contaminating RNA [14]. Subsequent plasmid preparations were purified using an RPM kit (Bio101).

The cloned PCR products (one to four clones per strain) were sequenced using purified plasmid DNAs, forward and reverse M13 sequencing primers and an ABI Prism sequencing kit (PE Applied Biosystems). Reaction products were analyzed using an ABI Prism 377 genetic analyzer. Sequences were aligned using DNAstar software and GCG. Oligonucleotide regions were analyzed individually using OLIGO version 5.0 (National Biosciences, Plymouth, MN, USA) to assess suitability as PCR primers.

2.5. Sequence analysis

Multiple sequences were aligned and edited using programs (SeqLab, Pileup, Pretty) in the Wisconsin (GCG) package [15]. For the Pileup program, a gap creation penalty of 5 and a gap length weight of 2 were used; fine adjustments were made manually after visual inspection. Phylogenetic analyses of aligned sequences were performed using the Farris algorithm for sequence addition with the heuristic search option of PAUP (version 4.0.0d55 for Unix) [16] run in SeqLab of GCG through Exceed (Hummingbird Communications Ltd., Toronto, Canada) X-windows. Analyses (PAUP) were performed with all characters unweighted. A phylogenetic tree was constructed using the original data set and 500 bootstrap data sets for the *G. occultum*/*G. brasilianum* tree (Fig. 4). Bootstrapping was used to calculate confidence indices for tree branches and thereby indicate the robustness of the tree structure. The tree shows only groupings with bootstrap values of more than 50%. Others were collapsed to polytomies in order to avoid misinterpretation of the low bootstrap supports.

3. Results and discussion

3.1. DNA amplification and sequencing

The ITS regions from the purported *G. occultum* strains IA702, FL703, CL700, CL383, and GR582, as well as other glomalean taxa and non-mycorrhizal control templates, reproducibly generated a 550–600-bp product from PCR amplification using ITS1*Kpn* and ITS4*Pst*. Sequences from these five *G. occultum* isolates were 94–99% similar in the 5.8S gene subunit regions (155 bp). Sequences in the ITS1 (135 bp) and ITS2 regions (230 bp) were more variable (82–98% and 71–99% similarity, respectively). Similarity between *G. occultum* sequences and those of the two *G. brasilianum* strains were greater in the 5.8 rDNA than in the ITS regions, ranging from 93 to 99% and 72 to 79%, respectively. The 94–99% similarity between ITS regions of *Glomus* strain GR582 and the two *G. brasilianum* sequences supports the view that this strain is more likely *G. brasilianum* than *G. occultum*. The rDNAs from other glomalean taxa showed much less similarity to either *G. occultum* or *G. brasilianum*. When aligned with sequences of the corresponding ITS and 5.8S gene regions from other glomalean taxa, (data not shown) many ITS regions appeared unique to the *G. occultum* isolates but only a few regions appeared useful in discriminating *G. occultum* from *G. brasilianum*. Fig. 1 shows sequence alignments for the rDNA ITS regions selected for development of the *G. occultum* primers GOCC56:GOCC427 and *G. brasilianum* primers GBRAS86:GBRAS388.

3.2. Selection and testing of GOCC and GBRAS oligonucleotides

Initial tests with combinations of GOCC primer pairs other than GOCC56:GOCC427 (Table 1) failed to yield amplified product from all isolates of *G. occultum* (results not shown). Reactions containing GOCC56 AND GOCC427 primed amplification of all seven isolates of *G. occultum* in our collection, but also yielded similar products from the four strains of *G. brasilianum* tested

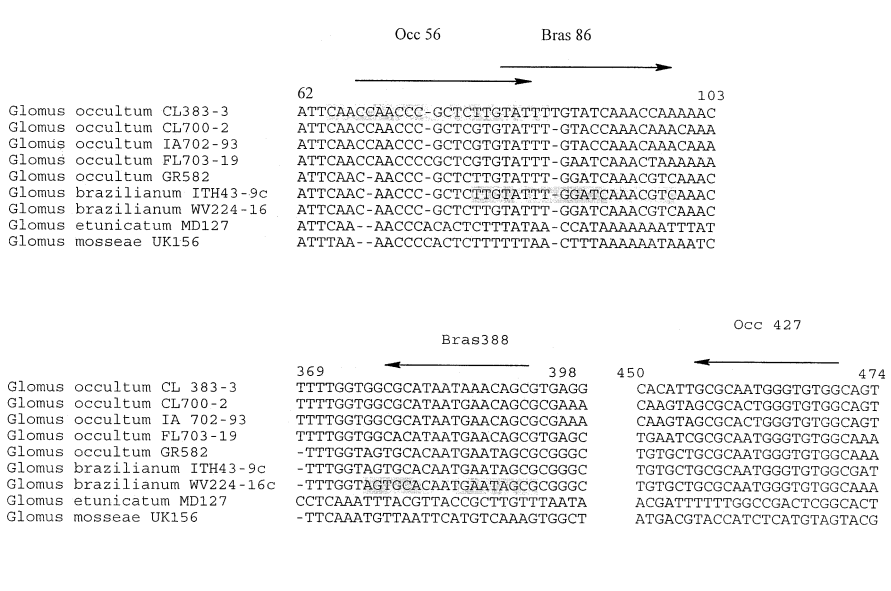

Fig. 1. Nucleotide sequences (5′ to 3′) and alignment of partial ITS regions from isolates of *G. occultum* with comparable regions from related fungal taxa. The sequences of the *G. occultum*-specific PCR primers (GOCC56 and GOCC427) and *G. brasilianum*-specific PCR primers (GBRAS86: GBRAS388) are indicated in bold type. Sequence numbering corresponds to the GenBank numbering system for *G. occultum* strain CL383-3 (GenBank accession number AF005064).

(Fig. 2A). However, no amplification of other *Glomus* species or other glomalean taxa was observed (Fig. 2). Subsequent cloning, nucleotide sequencing and alignment of the ITS rDNAs from *G. brasilianum* ITH43 and WV224 yielded ITS regions unique to *G. brasilianum*. Of the GBRAS primer pair combinations tested (Table 1) only the primer pair GBRAS86:GBRAS388 successfully amplified DNA from *G. brasilianum* strains without amplification of *G. occultum* DNA (Fig. 2B).

The PCR competency of all genomic DNA templates not amplified in reactions with GOCC56:GOCC427 and GBRAS86:GBRAS388 were verified by PCR assays primed by the ITS1*Kpn*:ITS4*Pst* primer pair; PCR products of the expected size (550–600 bp) were obtained from all those templates (results not shown). Thus, we conclude that primer pair GOCC56:GOCC427 is specific for *G. occultum* and *G. brasilianum* and that primer pair GBRAS86:GBRAS388 is highly specific only for *G. brasilianum*. Used in combination, these two primer pairs are capable of the detection and differentiation of DNA from *G. occultum* and/or *G. brasilianum*. This advances the specific detection of recently characterized ancestral lineages of the symbiotic Glomales that are undetectable by standard staining procedures when they have colonized the roots of host plants. In contrast to recently reported primer ARCH1311 [12], the primer pair GOCC56:GOCC427 only generates product with *G. occultum* and *G. brasilianum*, and the GBRAS86:GBRAS388 primer pair only generates product with *G. brasilianum*, not with *Acaulospora gerdemannii*, as reported for ARCH1311:ITS4.

The detection of *G. occultum* and *G. brasilianum* in root samples is an important intended use of these primers, therefore detection was tested by both direct and nested PCR. Detection of *G. occultum* in colonized *Z. mays* roots by PCR was achieved directly using the GOCC56:GOCC427 primer pair and root DNA extracts diluted 1:500 and 1:5000 (Fig. 3, lane A). PCR assays using comparable dilutions of root DNA extracts from uninoculated

Table 1
Oligonucleotide primers used in this study

Primer designation	Nucleotide sequence (5′-.....-3′)
GOCC-56	CAA CCC GCT CKT GTA TTT
GOCC-76	ACC CGC TCK TGT ATT TGW
GOCC-78	CCG CTC TTG TAT TTT GWA
GOCC-350	TYA TTR TGY RCY ACC AAA
GOCC-388	GCT GTT CAT TAT GYG CC
GOCC-390	GCG CTG TTA ATT ATG YG
GOCC-427	CCA CAC CCA KTG CGC
GOCC-429	TGC CAC ACC CAK TGC
GBRAS-76	ACC CGC TCT TGT ATT TGG
GBRAS-86	TGT AT TGG ATC AAA CGT C
GBRAS-473	CCC ATT GCG CAG CAC A
GBRAS-324	GCC TGC TGC GCA TTG C
GBRAS-364	CAC TAC CAA AAA CTR CCG
GBRAS-388	CGC TAT TCA TTG TGC ACT
GBRAS-421	TTT TAT TAC ACT CGC ATT G

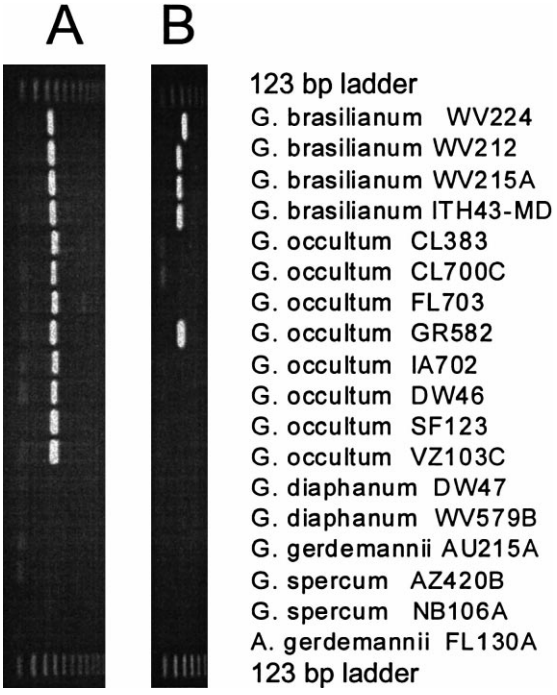

Fig. 2. Amplified products from PCR using *G. occultum*-specific or *G. brasilianum*-specific primers and genomic DNA preparations from spores of *G. occultum* and related fungal isolates. A: PCR products using primers GOCC56:GOCC427. B: PCR products using primers GBRAS86:GBRAS388.

plants and GOCC56:GOCC427 yielded no DNA products (Fig. 3, lane B). Nested amplification reactions were also tested using diluted corn root extracts and the primer pair ITS1*Kpn*:ITS4*Pst* in the first reaction and the primer pair GOCC56:GOCC427 in the second reaction (Fig. 3, lanes E,F). PCRs using ITS1*Kpn*:ITS4*Pst* and diluted root extracts from mycorrhizal or uninoculated roots both yielded products of approximately 550 bp (Fig. 3, lanes C and D, respectively). However, using the GOCC56:GOCC427 primer pair, the expected 400-bp *G. occultum* product was detected only when primary product was amplified from mycorrhizal root extracts (Fig. 3, lanes E,F).

The GBRAS86:GBRAS388 primer pair did not amplify DNA from *G. brasilianum*-infected roots either directly or indirectly using the primer pair ITS1*Kpn*:ITS4*Pst* in the first reaction (data not shown). However, it is unclear whether the roots used for these experiments were adequately infected (intraradical hyphae of *G. brasilianum* do not stain by conventional mycorrhizal root stains); additional root material from INVAM to repeat these experiments was unavailable. Further testing could potentially benefit from the use of recently discovered visualization of internal arbuscular structures with immunofluorescence microscopy using the anti-glomalin monoclonal antibody (personal communication, Sara Wright).

These results with *G. occultum* and *G. brasilianum* extend those from previous studies in which we identified and tested taxon-specific primers from the ITS region for

G. mosseae [10] and *G. etunicatum* [11]. Although the ITS region has proven useful for locating taxon-specific primers for these *Glomus* species, our preliminary results with *G. intraradices* strains suggest that there is too much intraspecific variation within the ITS regions of this broad group to find primers that would identify all strains without cross-reacting with closely related *G. etunicatum* and *G. claroideum* strains. Within the Gigasporaceae, our preliminary results suggest that genetic heterogeneity within the ITS regions of individual spores may be too high and interspecific heterogeneity too low to identify taxon-specific primers. However, it is likely that genus-specific primers can be identified for this important group. Among other AMF groups (*Scutellospora*, *Acualospora*, *Entrophospora*) there appears to be a variety of possible taxon-specific primer areas within the ITS but there are, as yet, too few sequences from these taxa to verify this prediction.

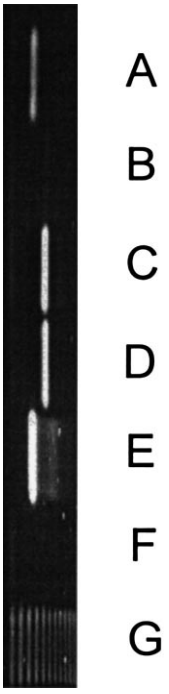

Fig. 3. Comparison of PCR products of rDNA amplified with primer pairs ITS1*Kpn*:ITS4*Pst* or GOCC56:GOCC427 and DNA preparations from inoculated and uninoculated corn roots. Lanes A, B: PCR products using GOCC56:GOCC427 and diluted DNA from roots of inoculated (lane A) and uninoculated (lane B) corn; lanes C, D: PCR products using ITS1*Kpn*:ITS4*Pst* and diluted DNA from roots of inoculated (lane C) and uninoculated (lane D) corn; lanes E, F: nested PCR products using GOCC56:GOCC427 primers to re-amplify PCR products from reactions using ITS1*Kpn*:ITS4*Pst* and diluted DNA from roots of inoculated (lane E) and uninoculated (lane F) corn. Lane G: 123-bp ladder. DNA preparations from roots were diluted 1/50 (lane A), 1/250 (lane C) or 1/500 (lanes B and D) in PCR-grade water. For nested PCRs (lanes E, F), aliquots of reactions using ITS1*Kpn*:ITS4*Pst* primers were diluted 1/1000 in PCR-grade water prior to use as templates for PCRs using GOCC56:GOCC427 primers.

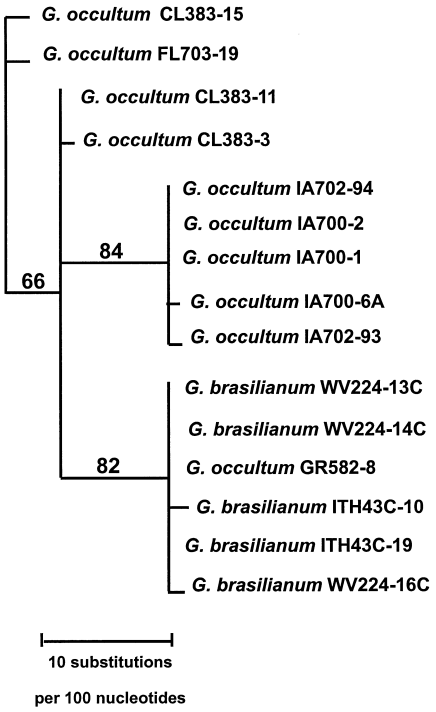

Fig. 4. The most parsimonious tree for 15 *G. occultum* and *G. brasilianum* rDNA sequences. The tree was inferred from a 277-nucleotide region spanning the 5.8S and a portion of the ITS2 region, using PAUP 4.0.0d55 with GCG SeqLab. Confidence limits of the branches were estimated by bootstrap analyses as described in Section 2; the values above the branch lines indicate the percent this group occurred in 500 bootstrap replicates. Horizontal lines are to the scale as shown. Dashed numbers after strain designations correspond to different clones.

3.3. Sequence analysis

Using the heuristic search and parsimony options from PAUP a phylogenetic tree was constructed using 15 *G. occultum* and *G. brasilianum* sequences (Fig. 4). This tree provides detailed insight on the *G. occultum* branch of previously published trees [4,11]; it shows the close but independent relationship of *G. brasilianum* to *G. occultum*, and the high degree of intraclonal and inter-isolate similarity in the 5.8S and ITS rDNA regions. Inclusion of these 15 sequences in a dataset used for a previously reported phylogenetic analysis of endomycorrhizal fungi [11], that included 39 sequences of the 5.8S region representing five genera (*Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, and *Scutellospora*), and 13 species, did not significantly alter bootstrap support for the ancestral branch comprised of *G. brasilianum* to *G. occultum* (tree not shown).

Acknowledgements

Robert Ames, Shannon Berch, Robert Lumsden, Jim Maddox, Joseph Menge, Joseph Morton, Stan Nemec, Yvonne Perez, Norman Schenck, Horace Skipper, Dorothy Watson, Sara Wright, and David Wilson, for gener-

ously contributed isolates (Table 1) used in this study. We thank Dorothy Watson and Deborah Kitt for exceptional technical assistance with pot cultures and spore harvests of the isolates, and Patrick O'Malley, Carl Patterson, Dexter Andrews, and Satyakam Patel for their help with DNA sequence analysis.

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